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Minireview

Accounting for Specificity in Receptor Tyrosine Kinase Signaling

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Many signal transduction mechanisms transmit information from the extracellular space or an intracellular compartment to the nucleus where they influence gene expression. This flow can be viewed metaphorically as the reverse of gene expression where information moves outward from the genome. However, the idea that signal transduction processes convey detailed amounts of information has been recently debated with respect to receptor tyrosine kinases (RTKs). Upon binding to their ligands, RTK molecules dimerize and undergo autophosphorylation on specific tyrosine residues. In turn, these phosphotyrosines and their adjacent sequences specifically recruit downstream signaling molecules via their SH2 domains, causing their subsequent activation. The activation of a single type of RTK thus leads to the activation of multiple intracellular signal transduction pathways. The naïve expectation would be that each pathway would have a distinct function. However, a number of studies have called this presumption into question. For example, a study of mutants of the platelet-derived growth factor receptor RTK led to the surprising conclusion that the downstream pathways are in fact redundant in terms of what genes they activate (Fambrough et al., 1999). I will first focus on these thought-provoking cell culture experiments before describing studies in whole animals that indicate that a significant amount of specific (i.e., nonredundant) information can be transmitted during RTK signaling.

To address whether distinct SH2 domain-containing signaling molecules send qualitatively different signals, Fambrough et al. (1999) created mutations in SH2 binding sites in the platelet-derived growth factor β receptor (PDGFRβ). NIH 3T3 cells were transfected with constructs encoding hybrid receptors with an intracellular domain from the PDGFRβ and the extracellular domain of the M-CSF receptor. Remarkably, simultaneous mutation of five SH2 binding sites, which drastically reduces the biological responsiveness of PDGFRβ function in cultured cells, had only modest quantitative effects on immediate-early gene inductions in response to the heterologous ligand M-CSF (which avoided activation of endogenous PDGF receptors) as measured by microarray hybridization. These data suggested that none of the sites tested (those that bound PI-3K, Ras-GAP, SHP-2, and PLCγ) were essential for gene activation. Since PGDFRβ is known to be phosphorylated on 11 sites (reviewed in Heldin et al., 1998), nonredundant roles in transcription may be played by signaling sites on the receptor that were not mutated in this study (Fambrough et al., 1999). Two caveats to the work are that saturating levels of ligand and overexpressed receptors were used (see review by Pawson and Saxton, 1999 for further discussion). Nonetheless, these highly provocative data raised the question of how much specific information is being transmitted by the distinct signaling molecules activated by RTKs, and suggested that the pathways may be functionally redundant (Figure 1A). If this view is correct, RTK pathways may function merely to activate transcription factors generically, whose activities would determine the consequences of signaling and whose identities would be specified by cell type (Figure 1B). As the authors themselves pointed out, it is important to extend these studies beyond laboratory-adapted tissue culture cells (Fambrough et al., 1999).

Engineered animals offer an appropriate test since in principle ligands and receptors are expressed at physiological levels and in the correct temporal pattern and tissue context. Knockin studies in mice of the PDGFRβ described above have yielded some evidence for distinct functions for SH2 docking sites in vivo. Mice containing a mutation in the PI-3K docking site were fully viable (in contrast to the null which dies during development due to cardiovascular, renal, and hematological defects), but exhibited a defect in interstitial fluid homeostasis (Heuchel et al., 1999). A double mutant in the PI-3K and PLCγ docking sites again resulted in viable mice, but these showed a defect in vascular cell fitness in chimeric mice (Tallquist et al., 2000). Studies in mice of other RTKs have also supported the view that the docking sites are not totally redundant. For example, mutation of PLCγ docking site of the essential FGFR1 RTK yields viable mice with a specific homeotic transformation (Partanen et al., 1998). The c-Kit RTK is required for normal hematopoesis, melanogenesis, and gametogenesis. Strikingly, mutation of the PI-3K docking site of this receptor results in a specific defect in gametogenesis (Blume-Jensen et al., 2000; Kissel et al., 2000). Two recent papers published in *Molecular Cell* inform further the issue of RTK specificity using engineered mice.

In experiments by Klinghoffer and colleagues, the intracellular domains of the α and β isoforms of the PDGF receptor (encoded by distinct genes) were exchanged to address the issue of RTK effector redundancy (Klinghoffer et al., 2001). The experiment asked whether replacement of the PDGFRα intracellular domain with that of β would result in a functional receptor as assayed in a knockin mouse. The reciprocal experiment was also performed. While the two isoforms are known to bind many of the same effectors such as Src, PI-3K, SHP-2, and PLCγ1, there are known distinctions. Unlike β, the PDGFRα intracellular domain binds the SH2-SH3 adaptor protein Crk; in contrast, β can bind Ras-GAP as well as a number of other signaling factors (reviewed in Heldin et al., 1998). Moreover, PDGFRβ promotes particular cytoskeletal changes, calcium mobilization, chemotaxis, and oncogenic transformation to a greater extent than the α isoform. Implicit in these experiments is the notion that complementation or its failure would be due to differences in the intracellular domains of the two isoforms.

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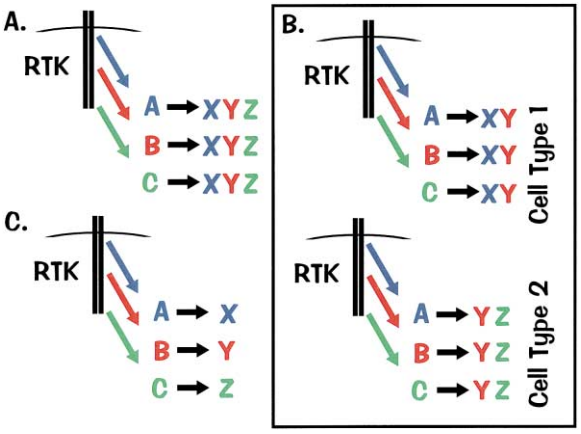

Figure 1. Models for Receptor Tyrosine Kinase Signaling Specificity

Shown is a generic RTK after ligand-induced dimerization, which causes the activation of three signaling pathways, "A," "B," and "C." "X," "Y," and "Z" represent distinct responses to these three pathways. In panel (A), the signaling pathways are redundant resulting in each activating the same responses, "X," "Y," and "Z." In panel (B), the pathways are redundant as in (A), except now, cell type refines the outcome of signaling such that outcomes "X" and "Y" occur in cell type 1 and outcomes "Y" and "Z" occur in cell type 2. In panel (C), the distinct signaling pathways lead to distinct responses.

The authors constructed two chimeras consisting of the ectodomain of one isoform and the intracellular domain of the other: αβ (i.e., α ectodomain, β intracellular domain) and βα (Klinghoffer et al., 2001). The αβ hybrid was then knocked into the α locus and the βα hybrid was knocked into the β locus. Remarkably, both chimera knockins were viable, suggesting that the differences in signaling capability of the intracellular domains of the two isoforms did not impact on overall survival. One interpretation of this result is that it is the shared effectors that bind both intracellular domains that are responsible for most of the receptors' functions. The result could equally be viewed as support for the redundancy of intracellular signaling pathways not shared by the two isoforms. Upon closer inspection, however, the authors discovered that all was not well with the mutant mice. The βα/βα homozygous mice suffered from moderate cardiac hypertrophy and βα/ hemizygous mice suffered from severe cardiac hypertrophy, glomerulosclerosis, and retinal detachment associated with a disorganized retinal vasculature. Interestingly, βα produces a less sustained phosphorylation of MAP kinase than wild-type, suggesting a potential signaling defect that could be responsible for these defects. Thus, while providing possible evidence for the redundancy model of RTK effector output, the careful analysis performed by the authors on the swaps suggests that there also is distinct information being transmitted (Klinghoffer et al., 2001). Because PDGFRβ contains a number of SH2 binding sites not present in PDGFRα, it is attractive to speculate that distinct effectors activated by PDGFβ are responsible for the defects of the βα chimera.

A second recent knockin mouse study also suggests that signaling downstream of a RTK does not involve solely redundant pathways (Maina et al., 2001). These authors focused on the Met RTK. Met binds the signaling proteins PI-3K, Src, Grb2, Shc, Gab1, and others primarily through two critical multifunctional docking sites (reviewed in Furge et al., 2000). Met is a receptor for hepatocyte growth factor (HGF), also known as scatter factor, and *met*/*met* null mice die during embryogenesis because of a defect in placenta formation and show loss of hepatocyes, decreased muscle, and defects in axon outgrowth and branching (see references in Maina et al., 2001). Because of the complexity of the docking sites, the authors attempted to create simpler alleles of the receptor that would direct signaling to specific pathways. The goal was to determine whether the effector pathways were redundant for the known functions of Met. The authors introduced two SH2 binding site mutations in the docking sites to bias them toward binding PI-3K (met^{2P}), Src (met^{2S}), or a change creating two Grb2 binding sites (met^{2G}). These mutations were knocked in at the Met locus and homozygous mice were created (Maina et al., 2001). Maina and colleagues then performed biochemical signaling studies of embryos and hepatocytes from these mice, which confirmed that the alleles were biased in the predicted manner. However, there were some exceptions, whose bases remain to be investigated. First, the met^{2G} allele displayed binding to all three effectors. Moreover, each of the mutant receptors displayed the ability to phosphorylate Gab1 and each exhibited some residual activation of the Ras-MAPK pathway.

As anticipated from its signaling properties, the met^{2G} allele behaved as wild-type in a variety of assays (Maina et al., 2001). In contrast, the met^{2P} allele was defective in a subset of *met* functions (hepatocye survival in vivo, placental development, and myoblast migration), and the met^{2S} allele exhibited several phenotypes as well (hepatocyte survival, myoblast migration, and axon outgrowth and branching). Thus, these knockin experiments suggest that the pathways activated by Met are not redundant, as the Src-biased and PI-3K-biased alleles could each only complement a subset of the defects seen in a mouse lacking *met* (Maina et al., 2001).

Admittedly, the results of studies described are difficult to compare directly because different receptors were analyzed, and the strategy for altering signaling was distinct in each case. The cultured cell study left a number of signaling sites in the PDGFRβ cytoplasmic domain intact and then mutated the remaining sites (Fambrough et al., 1999). The in vivo studies of PDGFR, FGFR1, and c-Kit assayed single or double docking site mutations (Heuchel et al., 1999; Partanen et al., 1998; Tallquist et al., 2000; Blume-Jensen et al., 2000; Kissel et al., 2000) or the differences known to occur between the two PDGFR isoforms (Klinghoffer et al., 2001). Again, there exist signaling effectors that bind both isoforms, making it difficult to interpret the wild-type phenotype of the αβ chimera PDGFR knockin. Studies of the Met RTK biased two multifunctional docking sites by site-directed mutagenesis (Maina et al., 2001). Moreover, immediate-early gene expression was used as the assay in one case (Fambrough et al., 1999), whereas the mouse studies utilized classical phenotypes. Expression profiling of the mutant mice would greatly inform the comparisons. Nonetheless, the lack of complementation seen in the mouse with particular mutations suggests that

signaling pathways induced by RTKs are not fully redundant at the phenotypic level (Figure 1C).

RTKs play key roles in *Drosophila* development and similar questions have been posed in this genetic model system. An intriguing study identified developmental mutations in the gene encoding the DSHC adaptor protein, a homolog of the mammalian SHC adaptor (Luschnig et al., 2000). *dshc* mutants are defective in signaling from the Torso and EGF RTKs but not from the Sevenless RTK, demonstrating that it functions within a subset of RTKs (Luschnig et al., 2000). Curiously, genetic analysis suggested that DSHC acts in parallel with other signaling proteins thought to also be activated by Torso; however, DSHC plays the most important role, that is, the redundancy with other signal mediators is partial. These data suggest that the pathways coming from the Torso RTK are not equivalent in vivo. Supporting this view is the striking observation that a homozygous null *dshc*/*dshc* mutation can completely suppress the effects of a dominant constitutive *Torso* allele [Tor^{4021}] (Luschnig et al., 2000). This result would not be expected if DSHC were fully redundant with other effectors of the Torso RTK. As with any dominant mutation, it is possible that the activated *Torso* allele elevates signaling above physiological levels. Nevertheless, the data are consistent with the results in the mouse whereby distinct molecules bound to RTKs can have distinct functions.

In *C. elegans*, signaling from the LET-23 EGF receptor activates the Ras-MAP kinase pathway to specify a number of cell types in the vulva, male tail, and elsewhere (reviewed in Sternberg et al., 1995). Appropriate responses are achieved in part by the expression of distinct transcription factors in distinct cell types (Tan et al., 1998). Thus, in cases in which the Ras-MAPK pathway is the effector, cell type differences provide the necessary specificity. The cytoplasmic domain of the receptor contains eight SH2 binding sites. Four of these sites (sites 4, 6, 7, 8) can activate through Ras-MAPK pathway via the SH2-SH3 adaptor protein SEM-5 (Lesa and Sternberg, 1997). A distinct site in LET-23, site 5, is also required for fertility, and it does not act via the Ras-MAPK pathway. The likely effector of site 5 was identified by an elegant genetic study in which suppressors of a mutation in the gene for the LET-23 ligand LIN-3 were isolated and characterized (Clandinin et al., 1998). The two genes identified *itr-1* and *lfe-2* were found to act downstream of *let-23* and to be required for ovulation similar to *lin-3* and *let-23*. Cloning studies revealed that *itr-1* encodes an inositol trisphosphate (IP_3) receptor and *lfe-2* encodes an IP_3-4 kinase (Clandinin et al., 1998). These data implicate the IP_3-calcium pathway as a tissue-specific effector of LET-23 in ovulation.

Two questions that can now be posed are why does the amount of information delivered by RTK signaling systems vary and, given the complexity of signaling, how do cells respond appropriately? The answer to the first question may be that in order for organisms with large numbers of cell types to respond to signals, it is advantageous to have a combination of specific information being relayed by a signaling system with further refinement by cell type. Combinatorial control that encompasses both signaling systems and transcription factors may be necessary in a complex eukaryote, such as a human, which has perhaps only six times the gene complement of a simple eukaryote, such as budding yeast (Goffeau et al., 1996; Lander et al., 2001; Venter et al., 2001). In yeast, signaling pathways can afford to be more dedicated or "hard-wired" such that each system transduces a specific signal to produce a specific output. Nonetheless, the question of precisely how cells respond appropriately to signals in the face of redundancy, cross-talk, and shared components in any system remains open.

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